Riboflavin is a component of the Na^+-pumping NADH–quinone oxidoreductase from *Vibrio cholerae*

Blanca Barquera, Weidong Zhou, Joel E. Morgan, and Robert B. Gennis*

Department of Biochemistry, University of Illinois, 600 South Mathews Street, Urbana, IL 61801

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Flavins are cofactors in many electron-transfer enzymes. Typically, two types of flavins perform this role: 5′-phosphoriboflavin (FMN) and flavin-adenine dinucleotide (FAD). Both of these are riboflavin derivatives, but riboflavin itself has never been reported to be an enzyme-bound component. We now report that tightly bound riboflavin is a component of the NADH-driven sodium pump from *Vibrio cholerae*.

Sodium-pumping NADH–quinone oxidoreductase (Na^+-NQR) is one of a number of enzymes that catalyze the oxidation of NADH and the reduction of ubiquinone (1). This is the only known case in which the redox reaction is coupled directly to pumping sodium ions across the cell membrane (2). The electrochemical sodium gradient generated by Na^+-NQR is a source of energy for cellular functions such as driving the bacterial flagellum and transporting nutrients into the cell (3, 4).

Na^+-NQR is the entry point for electrons into the respiratory chain of a number of marine and pathogenic bacteria (5–7). The enzyme has been isolated from several of these bacteria (8, 9) including *Vibrio cholerae* (10). The *nqr* operon from *V. cholerae* has been cloned and expressed in the parent organism (10), and the purified recombinant enzyme has been used in the current studies. The purified Na^+-NQR is an assembly of six polypeptide subunits (NqrA–F; ref. 10). Analysis of the polypeptide sequences indicates that five of the subunits contain transmembrane helices (11). There is no sequence homology to the subunits of the mitochondrial-type H^+-translocating NADH–quinone oxidoreductase (complex I).

Na^+-NQR contains several previously identified cofactors including a [2Fe-2S] center, a noncovalently bound FAD (Fig. 1), and two covalently bound 5′-phosphoriboflavins (FMNs) (refs. 8, 10, and 12; Fig. 1). The FAD, [2Fe-2S] cluster, and NADH-binding site all are thought to be associated with NqrF (11). It is probable that the FAD and the [2Fe-2S] center are the initial electron acceptors from NADH. The covalently bound FMNs are attached to NqrB and NqrC by phosphodiester linkages through threonine residues (10, 13). This mode of covalent flavin binding is unique to Na^+-NQR. In addition, Na^+-NQR contains an EPR-detectable radical that is observed in both the air-oxidized and dithionite-reduced forms of the enzyme (8, 10). EPR and electron nuclear double resonance spectroscopies have unequivocally established that the radical is present in a stoichiometry of 1 equivalent per mol of enzyme and is a neutral flavin-semiquinone in the oxidized form of the enzyme and an anionic flavin semiquinone in the NADH or dithionite-reduced forms of Na^+-NQR. (B.B., J.E.M., D. Lukoyanov, C. P. Scholes, R.B.G., and M. J. Nilges, unpublished data).

Materials and Methods

Enzyme Preparation. Recombinant Na^+-NQR from *V. cholerae* was purified according to a protocol described before (10). The *V. harveyi* Na^+-NQR was prepared as described (9).

Denaturation of Na^+-NQR and Analysis of the Denatured Enzyme by HPLC and Visible Spectroscopy. Na^+-NQR was denatured by using several different methods. Guanidine (6 M, pH 7) and 6M urea/2% SDS/100 μl of enzyme (80–100 μM protein concentration) were mixed with 900 μl of guanidine or urea, and the mixture was transferred to an Amicon centrifugation filter (10 kDa). The filtrate was recovered and analyzed by HPLC.

Boiling. The enzyme was incubated at 100°C for 5 min, and the denatured, precipitated protein then was removed by centrifugation. The supernatant was filtered through a 0.2-μm filter and analyzed by HPLC.

Cold TCA. The cold TCA had a final concentration of 5%. The supernatant after denaturation was analyzed by HPLC.

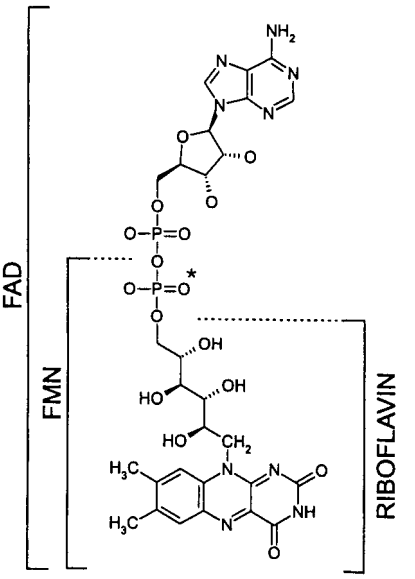

Fig. 1. Chemical structures of riboflavin, FMN, and FAD. The covalently bound FMNs are attached to the protein via ester bonds between their phosphate groups (marked * in the figure) and threonine residues in subunits B and C.

HPLC Running Conditions. A Microsorb-MV column (100-5-C18, 150 × 4.6 mm) was used to separate the flavins according to the method described (14). A 100-μl injection loop was used.

Fractions containing flavins (absorbance 450 nm) were collected and concentrated. Visible spectra of the initial sample, injected into the HPLC, and of the collected fractions were obtained by using a Cary (Sugarland, TX) 3E UV-visible spectrophotometer.

Mass Spectrometry. Collected samples from the HPLC run were concentrated by evaporation under a stream of nitrogen gas and then run on a Micromass Quattro I mass spectrometer by using

Abbreviations: Na^+-NQR, sodium-pumping NADH–quinone oxidoreductase; FMN, 5′-phosphoriboflavin; TCA, trichloroacetic acid.

*To whom reprint requests should be addressed. E-mail: r-gennis@uiuc.edu.

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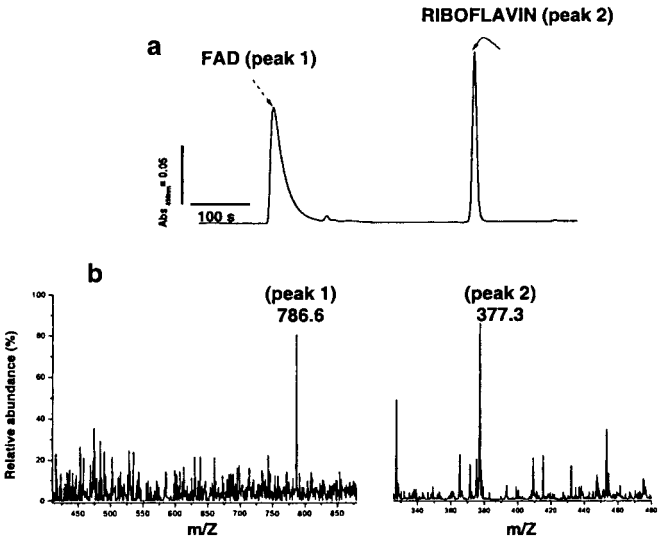

Fig. 2. HPLC separation (*a*) and mass spectrometric (*b*) analysis of the soluble flavin components extracted from Na^+-NQR after denaturation by 6 M guanidine-HCl. Shown is an HPLC elution profile of the extracted flavins monitored by the absorbance at 450 nm. The mass spectra of the two HPLC fractions are shown, corresponding to the expected masses of FAD and riboflavin. (*a*, *Inset*) UV-visible spectra of the material in each of the major HPLC peaks.

electrospray ionization. Samples were injected via a Rheodyne (Cotati, CA) loop-injection valve with a 10-μl loop. The flow system was water/acetonitrile at 15 μl/min. Data were acquired from 100–2,000 Da by using 10-sec scans.

Results and Discussion

Discovery of the flavin radical(s) in Na^+-NQR motivated a quantitative evaluation of the total flavin content of the enzyme. Denaturing the enzyme under conditions in which all the flavins would be oxidized revealed 3.7 ± 0.1-flavins per mol of enzyme based on the UV-visible absorption spectrum [ε_{450} = 12 $mM^{-1}{\cdot}cm^{-1}$ (15)]. Hence, there is a fourth flavin in Na^+-NQR in addition to the two covalent FMNs and the noncovalent FAD.

The additional flavin was found in the soluble fraction after denaturation of the enzyme. Na^+-NQR was denatured by using four different treatments: (*i*) boiling to precipitate the protein or by the addition of (*ii*) guanidine, (*iii*) urea, or (*iv*) cold trichloroacetic acid (TCA). When the enzyme was denatured by boiling or TCA addition, the soluble fraction was recovered simply by centrifugation. In the cases of guanidine and urea, the samples were passed through an ultrafiltration membrane (Amicon) with a molecular mass cut off of 10 kDa. The soluble fractions obtained by these different methods were analyzed by reverse-phase HPLC (14). The elution was followed at 450 nm, which is close to the absorbance maximum of oxidized flavins. The HPLC elution profiles were qualitatively the same for all the denaturation conditions used. Two distinct peaks are observed, as shown in Fig. 2*a*. The fractions corresponding to the two peaks have visible spectra characteristic of flavins (not shown). The first peak has the same retention time as FAD, whereas the retention time of the second peak matches riboflavin. These identifications were verified by mass spectroscopy (Fig. 2*b*), which showed that the major components of the two HPLC peaks have masses of 786.6 ± 1 and 377.3 ± 1 Da, respectively, corresponding to FAD (expected mass, 785.6 Da) and riboflavin (expected mass, 376.4 Da). For all the denaturing conditions analyzed, the ratio between the two peak areas was close to 1:1 (range of determined values was from 0.85 to 1), indicating that the riboflavin (Fig. 2*a*) is not a spurious contamination but a *bona fide*

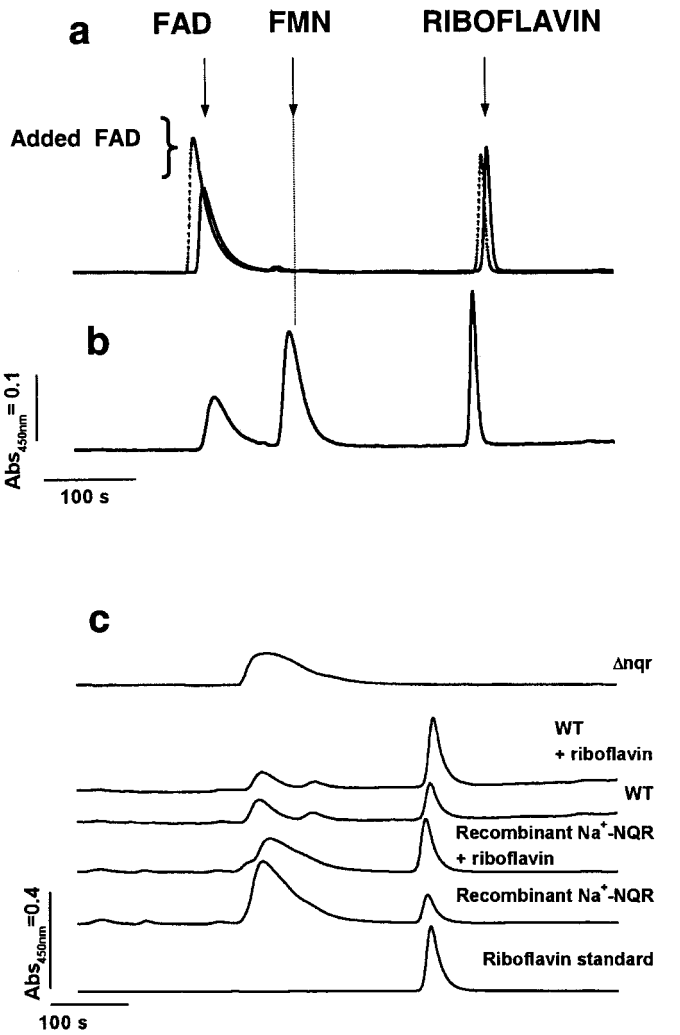

Fig. 3. HPLC elution profiles of low molecular weight fractions from denatured Na^+-NQR and from bacterial membrane preparations including internal standards and controls. (*a*) HPLC of flavins released from heat-denatured (boiled) Na^+-NQR with and without added FAD as an internal standard. Solid line, 10 nmol of flavins extracted from boiled Na^+-NQR; dashed line, same but with 5 nmol of FAD added before boiling the sample. There is a slight shift in the elution positions of the peaks that was within the range of variability observed between different samples. The FAD is not degraded, and the area of the riboflavin peak is not influenced by the inclusion of FAD to the sample before boiling. (*b*) Same as described for *a* but 5 nmol of FMN were added before boiling in place of FAD as internal standard. The FMN is not degraded, and the amounts of FAD and riboflavin are unchanged. (*c*) HPLC elution profiles of the soluble components from *V. cholerae* membrane preparations after cold-TCA denaturation showing the presence of riboflavin in membranes from wild-type (WT) cells and its absence in a strain where the genes encoding Na^+-NQR have been deleted. Riboflavin was used as both an internal and external standard as indicated in the labels.

component of the Na^+-NQR preparation. Incubation of the enzyme in the presence of 2 M KBr (overnight at 4°C) did not result in significant release of flavin from the enzyme, suggesting that the riboflavin is strongly associated with the enzyme.

Additional experiments were performed to address the possibility that the presence of tightly bound riboflavin is the result of enzymatic or chemical degradation of FAD or FMN during the preparation of the enzyme. To rule out these possibilities, exogenous FAD and FMN were combined with the Na^+-NQR sample before denaturation. In each case, 5 nmol of FAD or FMN were mixed with 10 nmol of the Na^+-NQR sample and

incubated for 1 h at room temperature or 4°C. The samples then were denatured by using each of the various methods described above.

Examples of the elution profiles from such experiments are shown in Fig. 3. When FAD was added (Fig. 3*a*), there was an increase in the amplitude of the FAD peak, whereas the amount of riboflavin remained constant. When FMN was added (Fig. 3*b*), a new peak corresponding to FMN was observed, but neither the FAD peak nor the riboflavin peak was altered. These results show that the presence of riboflavin in the samples is not likely to be caused by enzymatic or chemical cleavage of either FAD or FMN after protein purification. The results also confirm that Na^+-NQR does not contain any noncovalently bound FMN (12).

The possibility that the presence of riboflavin might result from the degradation of either FAD or FMN during the detergent solubilization or isolation of the enzyme was addressed also. The flavin content of the bacterial cell membranes was examined by directly extracting the membranes with cold TCA followed by HPLC analysis as described above. No riboflavin was present in the membranes of a strain from which the genomic *nqr* operon had been deleted (Δ*nqr* in Fig. 3*c*), which contrasts with the analyses of membranes from strains in which Na^+-NQR is expressed from a recombinant plasmid or strains expressing the genomic *nqr* operon without the recombinant plasmid. As shown in Fig. 3*c*, the presence of the riboflavin peak in the HPLC profile correlates with the presence of Na^+-NQR in the membranes, expressed either from the cloned, plasmid-borne *nqr* operon or the genomic *nqr* operon. These results clearly show that the presence of riboflavin is not an artifact of the enzyme preparation and is not peculiar to the recombinant enzyme. A similar analysis showed that riboflavin is present also in Na^+-NQR purified from the bacterium *Vibrio harveyi* (ref. 9 and data not shown), which demonstrates that the riboflavin cofactor is not limited to the enzyme from *V. cholerae*.

This report identifies riboflavin as a component in an enzyme (16). Up to now, riboflavin has been viewed only as a precursor for the synthesis of FMN and FAD cofactors. The phosphate(s) and adenylate groups of FMN and FAD provide contacts for the typical strong binding interactions between the protein and cofactor (17). In the absence of these interactions, the binding of the flavin to the protein would be more likely to involve the isoalloxazine ring. However, hydrogen bonding, stacking interactions, or covalent bonding with the isoalloxazine ring modulate the redox properties of the flavin and do not serve purely to anchor the cofactor to the protein (18). The interactions between the protein and riboflavin in Na^+-NQR might be expected, therefore, to have a strong influence on the redox properties of the riboflavin.

Any discussion of the functional significance of the riboflavin in Na^+-NQR must be purely conjectural. There are no data indicating either the location of the binding site or the role, if any, of riboflavin in the function of the enzyme. It is possible that riboflavin, which lacks the hydrophilic groups of FMN and FAD (Fig. 1), is buried within the membrane domain of the enzyme where it could play a role in long-range electron transfer (19) or the sodium translocation mechanism. These possibilities may be examined in further studies.

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